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Effect of Diocleinae lectins on bacteria and fungi planktonic and sessile cells

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Dissertação de Mestrado
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Trabalho realizado sob a orientação da
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e co-orientação da
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Abstract

Effect of Diocleinae lectins on bacteria and fungi planktonic and sessile cells

Microorganisms have been showing augmented resistance towards antimicrobials, being microbial resistance, nowadays, one of the biggest problems of public health. Thus, there is an increasing interest in the development of new strategies of microbial control, namely the ones based on natural products, especially from plants, such as lectins. So, it is of utmost importance to test new antimicrobial compounds, especially with a wide range, against bacteria and fungi. Therefore, the main aim of this thesis was to evaluate the effect of new lectins against both Gram-negative and Gram-positive bacteria, as well, as yeast. Moreover, these lectins were assessed against planktonic and sessile cells.

Four Diocleinae lectins: *Canavalia ensiformis*, *Canavalia brasiliensis*, *Canavalia maritima* and *Canavalia boliviana*, were used in this study. Their effect was assessed against two Gram-positive bacteria (*Staphylococcus epidermidis, Staphylococcus aureus*), two Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella oxytoca*) and two yeasts (*Candida albicans* and *Candida tropicalis*). The effect was evaluated on microbial planktonic growth, early-stage adhesion, on biofilm biomass and biofilm viable cells.

The four lectins showed different activities (inhibitory or stimulatory effect) on planktonic growth, early-stage adhesion and biofilm formation, for the same microorganism. In general, the inhibitory effect of lectins was most notable on planktonic growth than on biofilm formation. Although there are few differences in the inhibitory capacity of lectins, *K. oxytoca* can be considered the microorganism that suffered lower inhibition. Interestingly, the results demonstrated that activities of lectins tested were species-dependent, namely, their action was different between the two Gram-negative species, as well as the two Gram-positive and the two yeasts. This highlights that the interaction between the lectin and the cell is of high specificity.

So, in conclusion, due to the specificity of the lectins assayed, it could be of major interest to improve their potential (synergic effect) by using more than one at the same time or combined with conventional antimicrobial agents.

Resumo

Efeito de lectinas da subfamília *Diocleinae* em células bacterianas e fúngicas, no estado planctónico e séssil

A resistência exibida pelos microrganismos a agentes anti-microbianos representa um dos maiores problemas que a Saúde pública enfrenta na atualidade. Desta forma, tem-se denotado um aumento do interesse pelo desenvolvimento de novas estratégias para o controlo microbiano que passam pela procura de produtos naturais, especialmente oriundos de plantas, como as lectinas. A utilização de novos compostos antimicrobianos que possam ser usados contra uma vasta gama de bactérias e fungos, revela-se de extrema importância. Assim, o objectivo desta dissertação consistiu na verificação do efeito da aplicação de novas lectinas em bactérias Gram-positivas e Gram-negativas, bem como em leveduras. Além disso, estas lectinas foram testadas em células, quer no estado planctónico e quer no estado séssil.

Neste estudo foram utilizadas quatro lectinas da subfamília *Diocleinae*: *Canavalia ensiformis*, *Canavalia brasiliensis*, *Canavalia maritima* and *Canavalia boliviana*. O seu efeito foi avaliado em duas bactérias Gram-positivas (*Staphylococcus epidermidis, Staphylococcus aureus*), duas bactérias Gram-negativas (*Pseudomonas aeruginosa, Klebsiella oxytoca*) e em duas leveduras (*Candida albicans* and *Candida tropicalis*). A acção das lectinas foi analisada no crescimento planctónico, na adesão inicial, na biomassa de biofilme e no número de células cultiváveis dos biofilmes.

As quatro lectinas demonstraram atividades distintas (efeito inibitório e de estimulação) no crescimento planctónico, na adesão inicial e na formação de biofilme, para o mesmo microrganismo. Em geral, o efeito inibitório das lectinas foi mais notório no crescimento planctónico do que na formação de biofilme. Apesar da capacidade inibitória das lectinas não diferir muito entre os microrganismos, a bactéria *K. oxytoca* foi a menos sensível aos compostos usados.

Estes resultados demonstraram que a atividade das lectinas testadas se encontra dependente da espécie, sendo a sua ação diferente entre as duas espécies Gram-positivas, as duas Gram-negativas e as duas leveduras utilizadas. Corrobora-se, assim, a elevada especificidade existente na interação entre as lectinas e as células.

Em suma, dada a especificidade das lectinas testadas, pensa-se que estas poderão ter um potencial acrescido se utilizadas em conjunto ou combinadas com agentes antimicrobianos convencionais.

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List of Abbreviations

Aal	Lectin from *Araucaria angustifolia*
AHL	N-acyl homoserine lactone
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
CECT	Spanish Type Culture Collection
CFU	Colony forming unit
ConA	Concanavalin A from *Canavalia ensiformis*
ConBol	Lectin from *Canavalia boliviana*
ConBr	Lectin from *Canavalia brasiliensis*
ConM	Lectin from *Canavalia maritima*
CV	Crystal violet
DGL	Lectin from *Dioclea grandiflora*
DVL	Lectin from *Dioclea violacea*
EPS	Extracellular polymeric substance
ER	Endoplasmic reticulum
EuniSL	Lectin from *Eugenia uniflora*
GlcNAc	N-acetil-D-glucosamine
HHA	Lectin from *Hippeastrum hybrid*
LTA	Lipoteichoic acids
MIC	Minimum inhibitory concentration
MOA	Lectin from *Marasmium oreades*
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
PBS	Phosphate buffered solution
PHA	Lectin from *Phaseolus vulgaris*
POL	Lectin from *Pleurotus ostreatu*
PS	Polystyrene
Psl	Polysaccharide synthesis locus
PWM	Pokeweed mitogen
RIPs	Ribosome inactivating proteins
SBA	Soybean lectin

SCID	Severe Combined Immunodeficiency
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SSTI	Skin and Soft tissue infections
TFA	Lectin from *Trigonella foenumgraecum*
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WGA	Wheat germ agglutinin from *Triticum vulgaris*
WTA	Wall teichoic acid

Chapter 1 | Introduction

1.1. Contextualization and objectives

Today, bacterial and fungal infectious diseases are becoming hard to treat with conventional antimicrobial agents due to the development of resistance, being a serious health problem. This situation has promoted the search for new alternatives to treat infections and to control surface contamination.

Natural products, mainly plant-derived, are the most searched and studied compounds. Lectins are one of the many compounds extracted from plants, being proteins without catalytic function or immunological characteristics that bind specifically to carbohydrates. One of the largest and most studied families of lectins is those derived from leguminous plants.

Several lectins have been revealing to be able to inhibit bacterial and yeast pathogens, so, lectins may be important tools to control bacterial and fungal infectious diseases.

Until now, plant-based research has almost exclusively focused on planktonic microorganism, while biofilm forms, that are more resistant to antimicrobial agents and, consequently, more difficult to control, stay largely unexplored.

In this study, four plant lectins of Diocleinae subtribe (*Canavalia ensiformis* - ConA; *Canavalia brasiliensis* - ConBr; *Canavalia maritima* - ConM; *Canavalia boliviana* - ConBol) were screened for their effect on the growth, early-stage adhesion and subsequent biofilm formation of *Staphylococcus epidermidis* and *Staphylococcus aureus* (both gram positive bacteria), *Pseudomonas aeruginosa* and *Klebsiella oxytoca* (both gram negative bacteria) and for *Candida albicans* and *Candida tropicalis*.

1.2. Literature review

It is common that microorganisms attach to wet surfaces and form structures named biofilms. Biofilms are structured communities of surface-associated microbial cells enclosed in a self-produced polymeric matrix, forming sessile populations (1).

Biofilms still a concern in a broad range of areas such as food, environmental and biomedical fields. They can play important roles in bioremediation and fermentation facilities, facilitating stable operations, but on the other hand they are very problematic, particularly in food and health sector (2) (3). Many persistent and chronic microbial infections are related with biofilms, and occur essentially in hospital settings by contamination of indwelling medical devices. Medical devices are contaminated by the flora from the patient or health care personnel after touching contaminated environmental surfaces (2) (4). Such infections are difficult to diagnose and treat with conventional approaches, because biofilms have intrinsic mechanisms responsible for their highly resistance to antimicrobial agents (5).

The widespread use, and sometimes misuse, of antimicrobial agents leads to the development of microorganism resistance against conventional drugs (6). Microbial resistance is, nowadays, one of the biggest problems of public health. So, it is important to limit the spread of these microorganisms, limiting contamination of surfaces and medical devices, as well as finding appropriate antimicrobial agents for biofilm eradication (7).

Currently, the search for new environmental friendly and more effective strategies to control both planktonic and sessile microorganisms is becoming a priority. The research communities and pharmaceutical industries are searching new alternatives based on natural products with antimicrobial activity and low toxicity (8-11).

1.2.1. Microorganisms

Bacterial and fungal infectious diseases are a serious health problem being the lead cause of premature deaths all over the world (12-14).

Bacteria, such *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *K. oxytoca*, and yeasts from *Candida* genus, are some of the microorganisms responsible for human infections and important agents of nosocomial infections.

Staphylococcus epidermidis and *Staphylococcus aureus*

S. epidermidis and *S. aureus* are both Gram-positive bacteria.

S. epidermidis is a commensal skin bacterium and an opportunistic pathogen, being frequently isolated from infected medical devices, such as catheters (15) (16). Its infections promote an increase of global morbidity and mortality, especially in neonates (4).

S. aureus is the main etiological agent of numerous skin and soft tissue infections (SSTI). It remains the major human pathogen in hospital- and community- acquired infections causing diseases with high mortality rate (17). It is a common target for natural product drug screenings, because there has been observed an increase of antibiotics resistance, like methicillin-resistant *S. aureus* (MRSA) that presents a significant threat to public health (18) (19).

Klebsiella oxytoca and *Pseudomonas aeruginosa*

One of the most clinically important species of the genus *Klebsiella* is *K. oxytoca*. *Klebsiellae* are Gram-negative bacteria and opportunistic pathogens, being a frequent cause of severe diseases, such as urinary tract infections and pneumonia. *Klebsiella* infections are, essentially, associated with hospital care units (20) (21).

Another opportunistic Gram-negative bacterium, which is capable of causing fatal systemic diseases in humans, is *P. aeruginosa*. This pathogen infects patients, for example, when cutaneous barriers are breached and/or when immunologic system is compromised. Patients with respiratory diseases are commonly infected with *P. aeruginosa* and more than 90 % of them eventually die. *P. aeruginosa* is responsible for innumerous diseases, like pneumonia, SSTI, endocarditis, septicemia, among others, being the fourth leading cause of nosocomial infection and the main cause of hospital-acquired pneumonia (22) (23).

Candida albicans and *Candida tropicalis*

Candida species are the most common fungi involved in invasive human diseases, especially in immunocompromised patients (24).

C. albicans is generally considered to be an endogenous pathogen, but exogenous transmission (transmission from patient to patient by hands of health care personnel) is also verified (25). It is the main fungal pathogen agent found in humans, being responsible for both mucosal and deep tissue infections, such as infections of the skin, oral cavity, gastrointestinal tract, vagina and vascular system of humans (26) (27). Vaginal infections caused by *C. albicans* affect up to 75 % of women, at least, once in their life time (28).

C. tropicalis is the second most frequent *Candida* species, being the main cause of bloodstream and urinary tract infections, especially in hospitalized patients (29). It is

considered highly virulent and an important cause of infections in patients with cancer, especially leukemia (25) (26) (29).

1.2.2. Structure and components of the cell wall

Cell wall acts as a permeability barrier, maintains the cell shape and mediates the initial interaction between microorganism and the environment (24). Moreover, it is characteristic of both bacteria and fungi and has a protective function but, on the other hand, it is vulnerable to some antimicrobial agents which have the capacity to disturb the processes by which the wall is synthesized. During cell growth and proliferation, cell wall is continuously undergoing biosynthesis and extension, because it is a dynamic structure. This feature makes bacteria and fungi susceptible to antimicrobial agents which disturb the biosynthesis of the cell wall (6).

Cell wall structure of bacteria and fungi is different from each other, resulting in different susceptibilities to antimicrobial agents (6).

1.2.2.1. Bacterial cell wall

Gram-positive bacteria

As demonstrated in Figure 1, the basic structure of Gram-positive bacteria wall is quite simple. At least 50 % of wall mass is peptidoglycan, present in several layers. Peptidoglycan is organized in a cross-linked structure that provides a tough and fibrous fabric, giving shape and strength to the cell. This structure enables bacteria wall to resist a high internal osmotic pressure. Additionally, 30 to 40 % of the wall mass is due to an acidic polymer which is linked to peptidoglycan. This polymer is often a teichoic acid. There are two types of teichoic acids, namely wall teichoic acid (WTA) and lipoteichoic acid (LTA). Its acidic character guarantees that the cell surface is polar and carries a negative charge (6) (30).

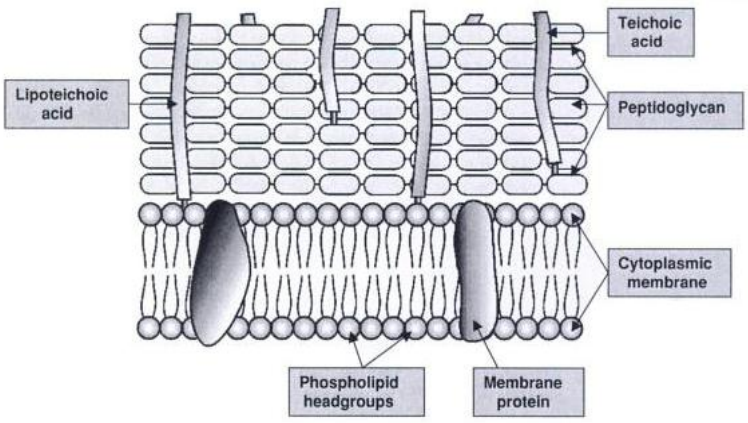

Figure 1. Representative scheme of Gram-positive cell envelope (cytoplasmic membrane and the cell wall (6)).

Teichuronic acid is synthesized, replacing teichoic acids, when phosphate supply to the cell is limited (6) (30).

Moreover, 5 to 10 % of the wall mass is related with the proteins present on the structure. Cell wall also contains neutral sugars, such as mannose, arabinose, rhamnose and glucosamine, and acidic sugars, such as glucuronic acid and mannuronic acid, which are present on the cell wall as subunits of polysaccharides. These proteins and polysaccharides often occur in the outer layers of the wall, being almost responsible for antigenic properties of bacteria (6) (30).

Gram-negative bacteria

The Gram-negative cell wall is far more complex than Gram-positive cell wall, as observed in Figure 2. Peptidoglycan is no longer the major component of the cell wall, being present only one or two layers of peptidoglycan (representing 5 to 10 % of the wall mass) (6) (30).

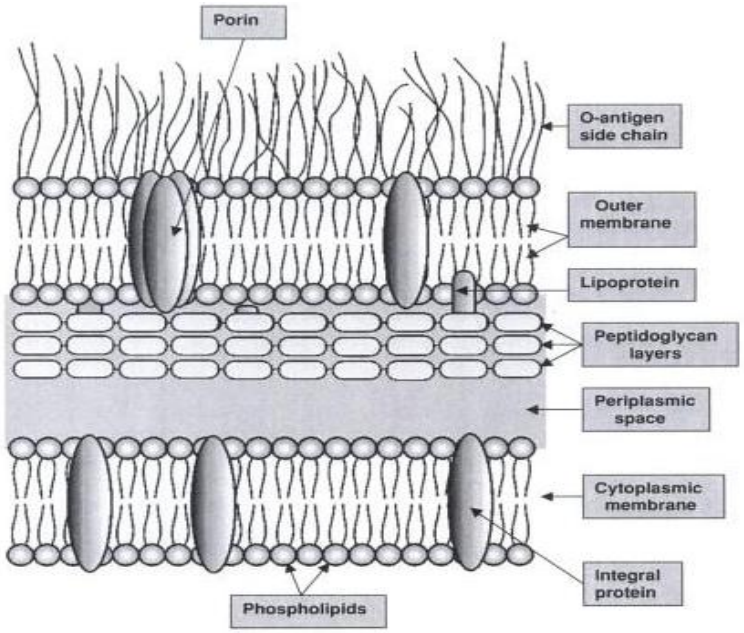

Figure 2. Representative scheme of Gram-negative cell envelope (cytoplasmic membrane and the cell wall) (6).

Just outside the cytoplasmic membrane, there is a layer called periplasmic space. This layer contains biochemical compounds, such enzymes, transport proteins, secreted materials and components of peptidoglycan. Immediately outside the periplasm, there is the peptidoglycan layer of the wall. It is much thinner than in Gram-positive bacteria, but still important to assure the wall strength (6) (31).

After peptidoglycan layer, the cell wall contains three main components, such as lipoprotein, outer membrane and lipopolyssacharides (30).

Lipoprotein is an important constituent of the outer membrane proteins. Only about one-third is linked to peptidoglycan, and the remaining, despite of being unattached, forms part of the membrane (6) (30) (31).

The outer membrane is composed by various compounds. It is basically a lipid bilayer with protein components. Lipopolysaccharide, phospholipids, fatty acids and proteins are the main constituents of this layer. The structure of lipopolysaccharide is complex and can vary from one bacterial strain to another. Lipopolysaccharides are exclusively on the outer surface of the outer membrane (6) (30) (32).

1.2.2.2. Fungal cell wall

Polysaccharides are the main component of fungal cell wall representing approximately 80 % of the cell wall dry weight (Figure 3). Proteins, lipids and various inorganic salts compose the others 20 % (33). The main polysaccharides found in diverse fungi cell wall are various forms of glucans and chitin, such as branched polymers of glucose containing β-1,3 and β-1,6 linkages (β-glucans), unbranched polymers of N-acetil-D-glucosamine (GlcNAc) containing β-1,4 bonds (chitin) and polymers of mannose covalently associated with proteins (24) (33) (34).

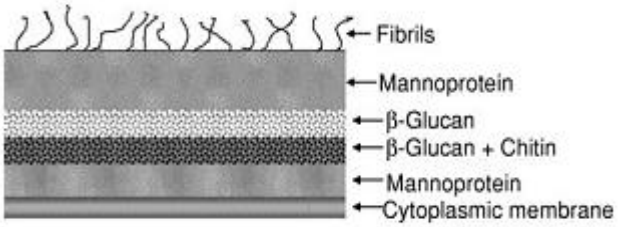

Figure 3. Representative scheme of fungal cell envelope (6).

Chitin and β-glucans (microfibrillar polymers) form the external skeleton representing the structural components of the cell wall. The skeleton provides strength to the cell (24) (33).

Although yeast cell wall composition is similar to the filamentous forms, hyphal cells contain at least three times more chitin than yeast cells (24) (33).

Proteins of the fungal cell wall are involved in permeability control, in recognition of other fungi, regulation of several processes and interaction with hosts (33).

1.2.3. Cell adhesion as virulence factor

Attachment of microorganisms, like bacteria, to tissues is the first step in the establishment of infection (18). Microorganism ability to adhere and consequently form a biofilm, which can cause an infection, is one of the major virulence factors (35).

Adhesion is a distinct process, but essential for biofilm development, that can be divided into two stages, namely the primary adhesion (docking stage) and the secondary adhesion (locking phase) (36).

Primary adhesion between bacteria/fungi and abiotic surfaces is usually mediated by nonspecific interactions, and between microorganism and living or devitalized tissues is mediated through specific molecular mechanisms (36).

This stage is reversible and dependent on the interaction between the microorganism cell surface and the conditioned surface. At an early stage, the organism has to achieve a critical proximity to the surface (generally < 1 nm). Then, adhesion depends on the sum of attractive or repulsive forces existent between the two surfaces, such as electrostatic and hydrophobic interactions, steric hindrance, Van der Waals forces, temperature, and hydrodynamic forces, among others. Electrostatic interactions are, generally, repulsive forces, because most bacteria and inert surfaces are negatively charged. Hydrophobic interactions are frequently the strongest of all long-range non-covalent forces, playing an important role in adhesion and consequently in microbial infections (3) (35) (36). The hydrophobicity of the conditioned surface can vary a lot, because it is dependent on the molecules in the conditioning film (15) (36).

Repulsion existent between two surfaces can be override by specific molecular interactions mediated by adhesins present on extracellular filamentous appendages (for example, pili and flagella) (3) (36). Pili are responsible to make cells more adhesive, so cells with these filamentous appendages adhere strongly to other cells or inorganic surfaces. Probably pili are capable to overcome the initial electrostatic repulsion barrier existent, being important to surface colonization (3).

Molecular mediated binding between specific adhesins and the surface is employed in the second stage of adhesion. Microorganisms consolidate the adhesion process producing exopolysaccharides. At the end, adhesion becomes irreversible being the microorganism firmly attached to the surface, without any physical or chemical interference (37) (38).

1.2.4. Biofilms

A biofilm is a well organized cooperating community of surface-associated microbial cells enclosed in a self-produced polymeric matrix (36) (37).

In response to environmental changes, microorganisms switch between planktonic growth and sessile form of life (2). Biofilm-associated microorganisms have a different behavior from planktonic organisms. They have reduced growth rates and ability to resist to antimicrobial treatments and host defenses, being a significant factor in microorganism virulence (4) (38) (39).

About 60 % of human infections results from biofilm formation on human mucosa (40). Biofilm are able to develop into implants and medical devices, such as catheters, cardiac valves, artificial joints, plates and screws, causing nosocomial infections (Table 1). These biofilms present in medical devices are usually a source of recurrent infections, which are notoriously more difficult to eradicate (2) (41). So, biofilm associated infections account for high morbidity and mortality rates (8) (42).

Table 1. Microorganisms frequently associated with biofilms on medical devices (adapted from (37))

Microorganism	Has been isolated from biofilms on
Candida albicans	Artificial voice prosthesis Central venous catheter Intrauterine device
Coagulase-negative staphylococci	Artificial hip prosthesis Artificial voice prosthesis Central venous catheter Intrauterine device Prosthetic heart valve Urinary catheter
Klebsiella pneumoniae	Central venous catheter Urinary catheter
Pseudomonas aeruginosa	Artificial hip prosthesis Central venous catheter Urinary catheter
Staphylococcus aureus	Artificial hip prosthesis Central venous catheter Intrauterine device Prosthetic heart valve

Depending on the device and its duration of action, biofilms can be formed by single or multiple species of microorganisms. For example, urinary catheter biofilm can be composed of single species at the beginning, but longer exposures lead to multispecies biofilms (38) (43). These biofilms on indwelling medical devices can include either yeast, Gram-positive or Gram-negative bacteria (38).

It is obviously that microorganisms have benefits from the biofilm mode of growth, such as a protective environment against harmful conditions in the host, ability to survive a nutrient limitation, osmotic stress, pH changes, anoxia, mechanical stress and exposure to antibiotics (2).

1.2.4.1. Biofilm development

Biofilm formation process may be divided into initial attachment, microcolony formation, maturation and dispersion, as shown in Figure 4 (2).

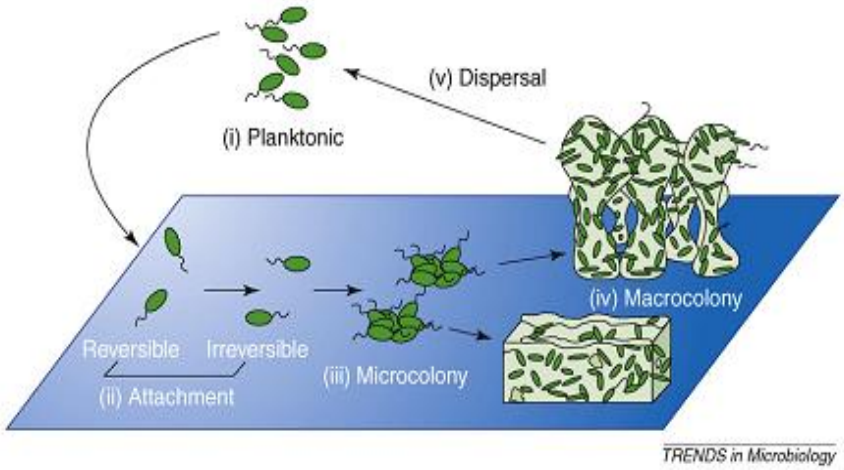

Figure 4. Schematic model of biofilm development processes (91).

On the second stage of adhesion, microorganisms are firmly attached to the surface, producing extracellular polymeric substance (EPS). Adhesion is closely followed by cell division and proliferation, raising microcolonies (1). This stage is a crucial step for biofilm development being the foundation upon which the mature biofilm will be built (36).

After establishment of microcolonies, the maturation process begins (2). The density and complexity of biofilm increase. Surface-adhered microorganisms actively replicate and extracellular components are generated through interaction between cells and molecules in immediate environment, raising glycocalyx (36).

Depending on environmental conditions, biofilms, for example from *P. aeruginosa,* can take a flat or mushroom-shaped structure (Figure 4). A flat structure is formed by means of twitching motility bacteria spread on the surface forming a confluent mat of cells. On the other hand, the migration of motile subpopulations of biofilm to the surface of a non-motile subpopulation (stalk) originates the caps, forming the mushroom-shaped structure (2). The growth potential and shape of a biofilm is dependent of the availability of nutrients, the perfusion of those nutrients to the cells within the biofim, the removal of waste, the internal pH, oxygen perfusion, osmolarity and carbon source (1) (36). Studies revealed that, when glucose is used as a carbon source, *P. aeruginosa* PAO1 forms biofilms with a mushroom-shaped structure, but when citrate, benzoate or casamino acids are used *P. aeruginosa* forms flat biofilms (44).

Fungi like *C. albicans* have the ability to grow with three different morphologies, such as yeast, pseudohyphae and hyphae. Hyphal growth is believed to be one of the most

important virulence factors (27) (28). Commonly, *Candida* species biofilms consist of two distinct layers, a thin, basal dense region of yeast cells, and an overlying thicker, but more open, hyphal layer, which raise a mushroom-shaped structure (41) (45). Dimorphism is not absolutely necessary for biofilm formation, but might be essential for the development of a spatially organized structure (41).

The last stage of biofilm development is dispersion (2). When a biofilm reaches a critical mass, a dynamic equilibrium is reached with the detachment of a subpopulation of biofilm cells (outermost layer of growth) which revert to a planktonic lifestyle. These organisms are free to escape from mature biofilm and colonize other surfaces (2) (36).

Cell to cell signaling, known as quorum sensing, has been demonstrated to play a role in cell attachment and detachment from biofilms, influencing biofilm maturation (37) (43).

Intracellular communication between microorganisms is usually carried out by bacteria/fungi products that are able to diffuse away from one cell and enter into another cell (43). Extracellular accumulation of signaling molecules synchronize expression of a set of genes which allow monitoring a factor such as cell population density (2).

Beyond preventing overpopulation, cell to cell signaling may alter distribution of microorganisms in the biofilm, alter protein expression and introduce new genetic in neighborhood cells, and incorporate microorganisms in biofilm (43) (45).

Studies revealed that farnesol acts as a quorum-sensing molecule inhibiting filamentation in *C. albicans*. Biofilm formation was almost completely inhibited when *C. albicans* cells was preincubated with high concentrations of farnesol. Moreover, supernatants recovered from mature biofilms inhibited filamentation of planktonic *C. albicans*, indicating that a morphogenetic autoregulatory compound (probably farnesol) is produced in biofilms (46).

On Gram-negative bacteria, such as *P. aeruginosa*, the best studied quorum sensing system is the LuxR-LuxI system, which uses an N-acyl homoserine lactone (AHL) as quorum sensing signal (47). Hentzer and colleagues (48) reported that a halogenated furanone compound (derivative of the secondary metabolites produced by *Delisea pulchra*) was able to interfere with AHL in *P. aeruginosa*. This interference affected the architecture of the biofilm and enhanced the process of bacterial detachment, leading to a loss of bacterial biomass from the substratum.

1.2.4.2. Biofilm resistance to antimicrobial agents

Biofilms are commonly highly resistant to antimicrobial agents (2). Several studies revealed that bacterial cells in a biofilm can become 10-1000 times more resistant to antibiotics than corresponding planktonic growing bacteria (1).

However, the mechanisms responsible for the resistance of biofilms to antimicrobial agents are not fully understood (41). Several mechanisms were proposed to explain the resistance of biofilms, including restricted penetration of drugs through biofilm matrix; the slow growth rates of biofilm; the appearance of resistant strains and phenotypic variants within biofilm populations, and expression of biofilm-specific genes (1) (2) (41).

Restricted penetration

Microorganisms in biofilms tend to be within the EPS matrix rather than on its surface. So, they are less accessible to antimicrobial agents. Thus, poor penetration of antimicrobial agents is suggested as one of the factors accounting for the resistance of biofilms (2) (41).

A reaction of the compound or its adsorption by the components of the biofilm matrix, result in slow diffusion rates and delayed penetration of antimicrobial agents within the biofilm. So, higher concentrations of antimicrobial agents are needed to kill biofilm cells regarding their planktonic counterparts (1) (2) (41).

For instance, *P. aeruginosa* biofilm formed on dialysis membranes delayed piperacillin diffusion suggesting that the biofilm matrix acts like a diffusion barrier to that antibiotic (2).

Slow growth rate

Biofilm cells grow slowly, mainly in mature biofilms, because of the limited availability of nutrients and the anaerobic conditions, particularly at the base of the biofilm (2) (36) (41). For example, beta-lactams and imipenem show low activity against mature biofilms, but they become more effective with younger biofilms (2). It is believed that oxygen limitations slow down microbial growth as it restricts the metabolic activity of the cells. Slow growth rate, oxygen limitation and low metabolic activity may be responsible for the antimicrobial resistance in biofilms. A slow growth rate is frequently accompanied by modifications in cell surface composition that could, in turn, affect the cell susceptibility to antimicrobial agents (2) (36) (41).

Persisters and phenotypic variants

Brooun and colleagues (49) observed that most of the *P. aeruginosa* biofilm cells were killed by a low concentration of antibiotics, but a subpopulation of cells survived even after a further antibiotic treatment with higher concentrations. These cells were called 'persisters' and it is thought that their programmed cell dead is disable. It is believed that physiological adaptive changes in persisters cells are responsible for the extraordinary survival ability of the biofilms (1) (2).

When microorganisms adhere to a surface and develop a biofilm they express an altered phenotype (1) (2) (41). Some researchers (49) (50) have started to identify genes that are repressed or activated in biofilms relatively to planktonic cells, with special interest in genes that may contribute to antimicrobial resistance. A recent study (51) demonstrates that genes encoding the two types of efflux pump present in *C. albicans* are upregulated during biofilm formation and development.

In conclusion, antimicrobial resistance in bacterial/fungal biofilms is a complex process that cannot be explained by a single molecular mechanism (41).

1.2.5. Natural antimicrobial agents

Natural products have been used since ancient times (52). For instances, penicillin was discovered in 1938, but its entirely development took place in twentieth century. With the discovery of penicillin, a new era began, being natural products replaced by synthetic or semi-synthetic substances. Antibiotics had an enormous success in controlling acute bacterial infections (6) (39) (52). However, bacteria, as well as fungal pathogens, have developed antimicrobial resistance against conventional antimicrobial agents due to widespread and indiscriminate use of these drugs (6).

The development of microbial drug resistance and drug-related toxicity has promoted the search for new alternatives, as natural drugs, to control, mainly, healthcare-associated infections (8-11). So, currently, there has been a widespread interest in the study of plant-derived compounds as alternatives for microbial control (11) (40) (53).

Natural compounds are generally accepted because of the perception that they are safe (as they have been used in folk medicine, since many years ago, for the prevention and treatment of infections and diseases) (40). Many components can be extracted from plants, such as lectins, extracts, essential oils and secondary metabolites (10) (54) (52).

1.2.5.1. Lectins

Lectins are proteins or glycoproteins with, at least, one carbohydrate or derivate binding site without catalytic function (non-enzimatic) or immunological characteristics (non-immune) (55-58). They have the capacity to bind, reversibly and with high specificity, to mono- and oligosaccharides without suffer any chemical modification. This distinguishes lectins from other enzymes and carbohydrate binding proteins, and makes them important tools for biotechnological and biomedical applications (56) (59-61).

Peumans and Van Damme (62) distinguished lectins in three major types based on their structure, namely "merolectins", "hololectins" and "chimerolectins". Merolectins (Figure 5) are small, single polypetide proteins, which are built exclusively of a single carbohydrate-binding domain. Because of its monovalent nature, they are incapable of precipitating glycoconjugates or agglutinating cells. Hololectins (Figure 5) have similar structural pattern as merolectins, but contain two or more carbohydrate-binding domains, which are very homologous. The majority of all known plant lectins are comprised in this group, because they have multiple binding sites, being capable of agglutinating cells or precipitating glycoconjugates (62) (63). Chimerolectins (Figure 5) are a group of fusion proteins which possess a carbohydrate-binding domain and an unrelated domain with another biological activity (for example catalytic activity) that acts independently of carbohydrate-binding domain. Chimerolectins can behave as merolectins or hololectins, depending of the number of sugar-binding sites (62) (63). For example, type 2 ribosome-inactivating proteins (RIPs) with two carbohydrate-binding sites on their B chain (e.g. ricin) agglutinate cells, whereas class I plant chitinases with a single chitin-binding domain do not (62) (64).

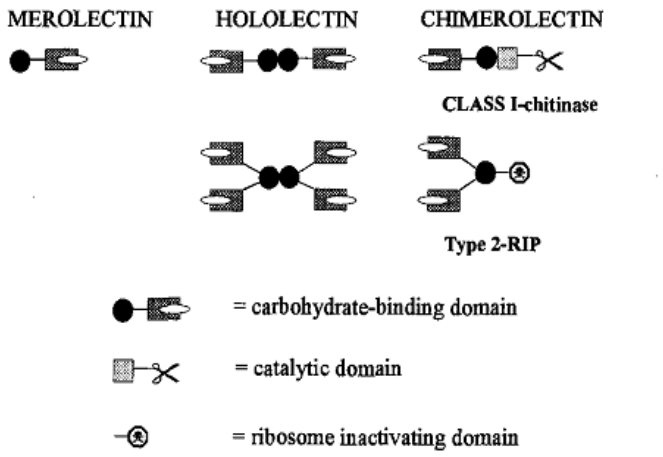

Figure 5. Schematic representation of three types of plant lectins: merolectins, hololectins and chimerolectins (62).

The first lectin discovered was ricin, a very toxic protein from *Ricinus communis*. It was extracted by Stillmark in 1888 (65). The first lectin isolated, sequenced and with tree-dimensional structure determined by x-ray crystallography, was ConA from *Canavalia ensiformis* (Figure 6). ConA is the best characterized plant lectin, with a high number of studies

(biochemical, biophysical and structural) (65) (66). ConA is a Diocleinae (Leguminosae) lectin and all Diocleinae lectins studied have many chemical, physic-chemical and structural properties in common. They share the same monosaccharide specificity for D-glucose and D-mannose and, despite their high similarity, they have distinct biological activities (9) (67) (68). The different biological activities are mainly due to changes in three parameters, such as binding specificity to complex carbohydrates, pH-dependent oligomerization state and the relative orientation of carbohydrate-binding sites. These differences in the described parameters among the lectins may result from specific amino acid replacements at key positions along their primary structures (66).

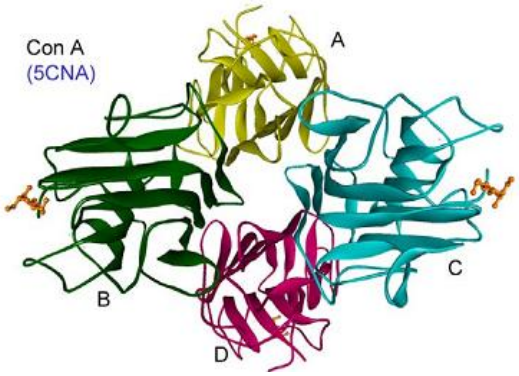

Figure 6. The X-ray structure of ConA (adapted from (68)).

Lectins may interact with carbohydrates through hydrogen bounds, Van der Waals forces, metal coordination and hydrophobic interactions. Essentially, hydroxyl groups on sugar molecules can serve both as a donor and an acceptor to cooperate in hydrogen bonds (69-71).

Most plant lectins studied are biosynthesized via the secretory pathway. Lectins are synthesized at ribosomes attached to the endoplasmic reticulum (ER). Then, lectins enter the lumen of the ER and are further transported through the Golgi apparatus (where lectins are modified by the glycosylational process), ending up in the vacuole (65) (72).

Lectin sources

Lectins are widely distributed in nature as they can be found in all kingdoms of life, ranging from animals, microorganisms (bacteria, yeasts and viruses) and plants (55) (58) (73).

Plants were the first discovered source of lectins, and remain the most frequently used (57).

Plant lectins can be framed in, at least, seven distinct families. The mainly four families of structurally and evolutionary related proteins are the legume lectins, the type 2 RIPs, the chitin-binding lectins, and the monocot mannose-binding lectins (74) (75). Legume lectins are the largest and most studied family of plant lectins. Some of the best known legume lectins are jackbean (ConA), soybean (SBA) and phytohemagglutinin (PHA) (76).

Additionally, smaller families (Cucurbitaceae phloem lectins, amaranthins and jacalin-related lectins) have also been characterized (74) (75).

Different families of plants, as well as different tissues of the same plant, can contain different lectins with different bioactivities and different carbohydrate-binding specificities (57) (77).

The richest sources of most lectins are the storage organs of plants, especially seeds. But also roots (*Urtica*, *Calystegia*), tubers or bulbs (*Allium*, *Tulipa*), bark (*Sophora*, *Robinia*) or leaves (*Aloe*, *Lactuca*) can contain significant amounts of lectins. The amount of lectin can be different from one plant species to other (65).

Biological activities and applications of lectins

Monosaccharides, such as mannose, galactose, N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylneuraminic acid, are typical constituents of surfaces of eukaryotic cells. Almost all lectins have high specificity for the sugars describes above, being this fact relevant for biological activities of lectins. The binding of lectins is dynamic and reversible. The association constant of lectin-monosaccharide interactions is relative low (in millimolar range) and lower than lectin-oligosaccharide interactions, however it is often highly selective. This is due to the fact that lectins exhibit an exquisite specificity for di-, tri-, and tetrasaccharides (76).

Lectins ability to bind carbohydrates with considerable specificity makes them valuable tools in scientific research, particularly in clinical, biochemical and biotechnology studies (76) (78). Some examples of common applications of plant lectins as tools in basic and medical sciences are demonstrated in Table 2.

Table 2. Common applications of plant lectins as tools in basic and medical sciences (adapted from (65))

Biochemistry
Detection of defined carbohydrate epitopes of glycoconjugates in blots or on thin-layer chromatography plates
Purification of lectin-reactive glycoconjugates by affinity chromatography
Glycan characterization by serial lectin affinity chromatography
Glycome analysis (glycomics)
Quantification of lectin-reactive glycoconjugates in enzyme-linked lectin-binding assays (ELLA)
Quantification of activities of glycosyltransferases/glycosidases by lectin-based detection of products of enzymatic reaction
Cell biology
Characterization of cell surface presentation of glycoconjugates and their preceding intracellular assembly and routing in normal and genetically engineered cells
Analysis of mechanisms involved in correct glycosylation by lectin-resistant cell variants
Fractionation of cell populations
Modulation of proliferation and activation status of cells
Model substratum for study of cell aggregation and adhesion
Medicine
Detection of disease-related alterations of glycan synthesis
Blood group typing and definition of secretor status
Quantification of aberrations of cell surface glycan presentation, e.g. in malignancy
Cell marker for diagnostic purposes incl. infectious agents (viruses, bacteria, fungi, parasites)
Mitogenic stimulation of lymphocytes
Purging of bone marrow for transplantation

- **Blood typing**

Lectins are frequently used for applications based on agglutination or precipitation reaction (76). ConA is capable of agglutinates erythrocytes and yeasts, as consequence of lectin interaction with carbohydrates on the surface of the cells. Lectins with ability to agglutinate erythrocytes are named as hemagglutinins (79).

Most legume lectins have hemagglutinating activity and most of them also exhibit blood type specificity, so they can recognize the difference in blood group antigens having the carbohydrate epitopes (ABO, Le, Ii, T, Tn, Forssman antigens, etc.). Studies revealed that extracts of the asparagus pea (*Lotus tetragonolobus*) only agglutinated blood type O erythrocytes, whereas crude extracts of the lima bean (*Phaseolus limensis*) agglutinated only blood type A erythrocytes. The lectin from *Dolichos biflorus* is used to distinguish between A_1 and A_2 blood subgroups (63) (76) (79).

Hemagglutinins which exhibit blood type specificity played a key role in early investigations on the structural basis of the specificity of the antigens associated with the ABO blood group system (79).

- **Antibacterial and antifungal activity**

Owing to the physiological functions of plant lectins in nature (plant's defense), antibacterial and antifungal activity of lectins has been intensively discussed (62). Interactions between lectins and human pathogenic microorganisms were reported since many years ago (58). Lectins are extensively used in microbiology and parasitology domain for identification of the agent structures and with protective effect against infection (66).

Recently, several studies were carried out to verify whether lectins have some antibacterial and/or antifungal activity. Cavalcante and colleagues (80) verify that *Staphylococcus mutans* growth was affected by ConBol, ConBr and ConM lectins. Oliveira and colleagues (58) demonstrated that a lectin from *Eugenia uniflora* (EuniSL) strongly inhibited the growth of *S. aureus*, *P. aeruginosa* and *Klebsiella sp*. and moderately inhibited the growth of *Bacillus subtilis*, *Streptococcus sp*. and *Escherichia coli*. Santi-Gadelha and colleagues (77) used a lectin from *Araucaria angustifolia* (Aal) against Gram-positive and Gram-negative bacteria concluding that this lectin had stronger antimicrobial activity. They observed, through electron microscopy, the presence of pores and severe disruption of the Gram-positive bacteria membrane and substantial bubbling in Gram-negative cell wall, besides some destroyed bacteria.

Lectins from the seeds of *Phaseolus vulgaris* and *Pisum sativum* (81), from the mushroom *Astragalus mongholicus* (82), and from the pepper seed *Capsicum frutescens* (83),

have been reported to have antifungal activity. Antifungal properties have also been found from the pepper seeds *Capsicum annuum* by Kuku and colleagues (83), and from a chitin-binding lectin from *Talisia esculenta* (84). This latter lectin had antifungal activity against *Fusarium oxysporum*, *Saccharomyces cerevisiae*, etc.

Charungchitrak and colleagues (57) demonstrated that a lectin from the seeds of *Archidendron jiringa* had antifungal activity against, for example, *Exserohilum turcicum* and *Colletotrichum cassiicola*, and antibacterial activity against Gram-positive and Gram-negative bacteria, such as *P. aeruginosa* and *S. aureus*. These results indicated that some lectins are able to inhibit both bacteria and yeasts pathogens (57).

- **Mitogenic stimulation**

The first lectin found with mitogenic activity was PHA (lectin from *Phaseolus vulgaris*), but nowadays there are several lectins with this capacity. A mitogenic lectin possesses the ability to stimulate lymphocytes to undergo mitosis. The activation of lymphocytes occurs as a result of binding of lectins to cell surface sugars (79) (85).

Lectins such as PHA and ConA are able to stimulate T lymphocites (T cells), while pokeweed mitogen (PWM) is able to stimulate both T and B cells. This stimulation by lectins provides an easy and simple means to assess the immunocompetence of patients which suffer from a diversity of diseases, including AIDS (Acquired Immune Deficiency Syndrome), and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations (76).

- **Antiproliferative activity and cancer diagnosis**

Lectins have been shown to be of great interest since they have been reported as potential anticancer agents. They have been tested as adjuvants in tumor treatment and in diagnosis (66).

For example, lectins like *Agaricus bisporus*, *Tricholoma mongolicum* and *Volvariella volvacea* have antiproliferative and antitumor action. Wang and colleagues (86) demonstrated that lectin from *Pleurotus ostreatus* (POL) when administrated in mice promotes a dramatic shrinkage of sarcoma S-180 and hepatoma H-22 cells, having a potent antitumor activity. A similar situation was observed when three cancer cell lines (HeLa, L929 and EATC) were treated with ConA and PHA-P lectins. These lectins inhibited cell proliferation and caused damages on cell lines used, being the effect of PHA-P stronger than ConA. Cells showed morphological changes (damages), such as dilatation of mitochondria, cytoplasmic vacuolation, rounded cells and cell aggregation (87).

Development of cancer cells seems to be associated with changes in cell surface sugars which lead to the assumption that high susceptibility to agglutination by lectins was a property shared by malignant cells. Wheat germ agglutinin (WGA) and ConA have the ability to selectively agglutinate neoplastic cells (79). Because malignant cells display altered surface glycoproteins, exogenous lectins tagged with the killer T cells can promote the lyses of tumor cells, controlling tumor (Figure 7). Also, lectins present on the surface of malignant cells are able to binding exogeneous sugars which contain molecules and are capable of internalizing them by endocytosis, having a potential use in cancer treatment strategies (63).

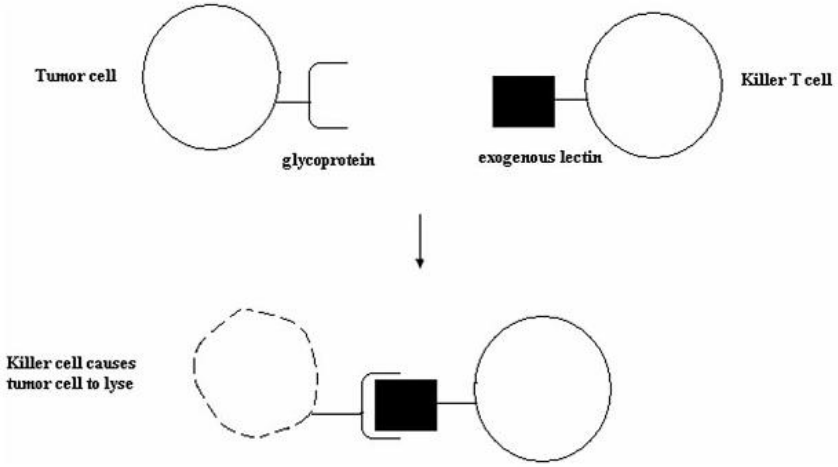

Figure 7. Exogenous lectin attached to the T cell targeting the altered glycoprotein present on tumor cell surface, promoting the lyses of the cell (63).

- **Bone marrow transplantation**

Some lectins have been used in organ transplantation applications. Selective agglutination by SBA allows separation of B and T mouse splenocytes (bone marrow). Since 1981 SBA lectin is used for purging human bone marrow for transplantation. It is employed frequently for transplantations into children born with severe combined immune deficiency (SCIDs or "bubble children" – they are highly susceptible to microbial infections). About 75 % of the hundreds of children transplanted after SBA purging of bone marrow have been cured and lead a normal life. SBA-purged bone marrow was also used experimentally for treatment of end stage leukemia patients, as an alternative to other techniques for T cell depletion, such as monoclonal antibodies (76) (85).

Chapter 2 | Materials and Methods

2.1. Lectins origin

The lectins *Canavalia ensiformis* - ConA; *Canavalia brasiliensis* - ConBr; *Canavalia maritima* – ConM and *Canavalia boliviana* - ConBol were gently provided by Professor Benildo Cavada from BioMol-Lab (Laboratory of Biologically Actives Molecules, Department of Biochemistry and Molecular Biology, Federal University of Ceará - Brazil).

2.2. Lectins stock solution

Phosphate buffered solution (PBS) was prepared in sterilized water being added 8 g/L of sodium chloride (NaCl), 0.2 g/L of potassium chloride (KCl), 0.3 g/L of potassium dihydrogen phosphate (KH_2PO_4) and 1.15 g/L of disodium phosphate (Na_2HPO_4). The pH was adjusted to 7.0. Before being used, this solution was autoclaved at 121 ˚C for 15 minutes and reserved at room temperature.

The Diocleinae lectins were solubilised in PBS previously prepared, at 37 °C and 120 rpm for 3 h, in order to obtain stock solutions with a concentration of 1 mg/mL. After that, the solutions were filtered through a 0.22 µm cellulose filter and preserved at 4 °C.

Immediately prior to experiments, the lectin stock solution was diluted in Tryptic Soy Broth (TSB; Liofilchem) for bacteria, and in Sabouraud Dextrose broth (SDB; Liofilchem) for yeasts, to obtain the concentrations of 62.5, 125, 250 and 500 µg/mL of lectin. Then, bacteria and yeasts suspensions (after its concentrations has been adjusted) were added to the diluted solutions of lectins, obtaining the final concentrations of 31.2, 62.5, 125 and 250 µg/mL of lectins used in experiments.

2.3. Microorganisms and culture conditions

The microorganisms used in the experiments are all reference strains from American Type Culture Collection (ATCC) and Spanish Type Culture Collection (CECT). This included two Gram-positive bacteria (*Staphylococcus epidermidis* CECT231 and *Staphylococcus aureus* ATCC), two Gram-negative bacteria (*Klebsiella oxytoca* ATCC13182 and *Pseudomonas aeruginosa* ATCC10145), and two yeasts (*Candida albicans* ATCC1472 and *Candida tropicalis* ATCC750).

Bacteria were preserved at – 80 ˚C in TSB supplemented with 20 % glycerol. Yeasts were preserved at – 80 ˚C in SDB supplemented with 10 % glycerol.

Prior to each experiment, bacteria and fungi cells were grown, respectively, on Tryptic Soy Agar (TSA; Merck) and on Sabouraud Dextrose Agar (SDA; Liofilchem) plates for 24 h, at 37

˚C. These media were previously prepared and autoclaved at 121 ˚C for 15 minutes and reserved at room temperature.

After 24 h of growth on solid media, an isolated colony of each microorganism was removed and used to inoculate 20 mL of TSB or SDB, and incubated for 18 h, at 37 °C, under constant agitation of 120 rpm.

Prior to use, the bacteria and yeasts suspensions were adjusted to a final concentration of 2 x 10^8 cells/mL (for early adhesion characterization) and 1 x 10^6 cells/mL (for planktonic and biofilm growth tests). Bacterial suspensions were adjusted through the measurement of the optical density (OD) at 640 nm and calibration curves, previously determined for each bacterium. The yeasts inocula were adjusted using a Neubauer chamber.

2.4. Lectin antimicrobial activity

All bacteria and yeast suspensions were adjusted to 1 x 10^6 cells/mL, and then 100 µL of each suspension were transferred to a 96-well tissue culture plates (Orange Scientific), previously prepared with 100 µL of TSB or SDB supplemented with a dose of each lectin (described on section 2.2). These plates were incubated during 24 h at 37 °C and 120 rpm.

Microbial growth was determined through measurement of optical density at 640 nm of the liquid content of each well using a microtiter plate reader, being the bacteria and yeast planktonic growth results presented as $OD_{640\ nm}$.

Negative control, wells contained culture medium only, was also performed in order to determine whether the tested medium interfere with the measurement of planktonic growth.

2.5. Lectin effect on early-stage adhesion

To verify the effect of lectins on bacteria and yeast early-stage adhesion, the cells were put in contact with a range of concentrations of all lectins, during 2 h at 37 °C and 120 rpm. Each bacteria and yeast suspensions were previously adjusted to 2 x 10^8 cells/mL. Then, 500 µL of each cell suspension was transferred to a 24-well tissue culture plates (Orange Scientific), containing 500 µL of TSB or SDB per well, supplemented with a dose of each lectin (described on section 2.2).

After 2 h of contact, the content of each well was removed, the adhered cells were washed twice with sterilized water, being the biomass quantified by crystal violet (CV) staining, according to Stepanovic and colleagues (88). For that, the plates containing the adhered cells were left to air dry for 30 min, and 1 mL of 98 % methanol were transferred to each well in

order to fix the remaining attached cells, for 15 min. Afterwards, the plates were emptied and left to air dry. The fixed cells were stained with 1 mL of CV (Gram's staining; Merck) per well, for 5 min. After this staining step, plates were washed with running tap water, air dried, and filled with 1 mL of 33 % (v/v) of acetic acid (Merck) in order to resolubilize the CV bound to the adherent cells. After that 200 µL of each well was transferred to a 96-well tissue culture plates and the quantitative analysis of adhered cells was performed through the measurement of optical density at 570 nm of each well using a microtiter plate reader, being the results presented as OD_{570nm}. Negative control, wells contained culture medium only, was also performed in order to determine whether the tested media and the plate material could adsorb CV and interfere with the adhered cells quantification.

2.6. Biofilm prevention

To assess whether the presence of the lectins could interfere with the establishment of biofilms by bacteria and yeasts on polystyrene (PS) surfaces, biofilms (24 h) were allowed to form in the presence of a range of concentrations of all lectins. Biofilms were developed using the microtiter plate test developed by Stepanovic and colleagues (88). Briefly, 100 µL of each bacteria and fungi suspensions, previously adjusted to 1 x 10^6 cells/mL, were transferred to each well of a 96-well tissue culture plates (Orange Scientific) containing 100 µL of TSB or SDB per well supplemented with a dose of each lectin (described on section 2.2).

After 24 h of biofilm growth, the content of each well was removed; biofilms were washed twice with sterilized water and reserved for further characterization.

2.6.1. Biofilm biomass quantification

The biomass of the biofilms was quantified by a staining method, as described for early adhesion. For quantification of the biofilm mass, the quantities of methanol, CV and acetic acid were 200 µL per well. After resolubilize the CV bound to the adherent biofilm, the quantitative analysis of biofilm mass was performed through the measurement of optical density at 570 nm of each well using a microtiter plate reader, being the biofilm mass presented as OD_{570nm}. Negative control, wells contained culture medium only, was also performed in order to determine whether the tested media and the plate material could adsorb CV and interfere with the biofilm biomass quantification.

2.6.2. Biofilm viable cells enumeration

In order to determine the number of viable biofilm-entrapped cells, biofilm suspensions were prepared.

For the bacterial biofilms, 200 µL of sterilized water were added to each well, being the biofilms detached by ultrasonic bath in a Sonicor SC-52 (Sonicor Instruments) operating at 50 kHz, during 6 min. The yeasts biofilm cells suspensions were obtained by completely scraping the adhered biomass from the wells, through the use of a yellow tip, into 200 µL of sterilized water. Afterwards, the biofilm suspensions of each 2 wells per condition were collected and gently vortexed for 1 min to disrupt possible cell aggregates (these parameters were previously optimized in order to promote the complete removal of all the biofilm-attached cells without lysis).

Biofilm suspensions were then serially diluted, plated on TSA (bacterial suspensions) or on SDA (yeasts suspensions), and incubated at 37 °C in an aerobic incubator for 24 h. The number of colony forming units (CFU) was enumerated, being the biofilm cell numbers presented as $\log_{10}$ (CFU/cm^2).

2.7. Statistical analysis

Statistical analyses were performed by PASW Statistics. The method used was one-way ANOVA with Tukey *post hoc* test. Data were obtained in triplicates from, at least, three separate experiments. The results were presented as mean ± standard deviation (SD). A P-value <0.05 was considered indicative of statistical significance.

Chapter 3 | Results

3.1. Bacterial and yeast planktonic growth

After 24 h in contact with lectins, bacteria and yeast growth was determined through measurement of optical density at 640 nm. The effect of lectins on microbial planktonic growth is shown in Figure 8.

For *P. aeruginosa* (Figure 8a) and *K. oxytoca* (Figure 8b), both Gram-negative bacteria, the higher concentration of the all lectins, with the exception of ConBol, showed inhibitory activity ($p<0.05$).

Curiously, ConA and ConBol stimulated ($p<0.05$) the growth of *S. aureus* (Figure 8c). ConBr did not demonstrate significant differences compared to the control ($p>0.05$). In opposition, higher concentrations of all lectins had antimicrobial activity ($p<0.05$) against *S. epidermidis* growth (Figure 8d).

All lectins showed inhibitory activity ($p<0.05$) for *C. albicans*, but they enhanced the growth ($p<0.05$) of *C. tropicalis*, with exception of ConBr which had no significant effect ($p>0.05$).

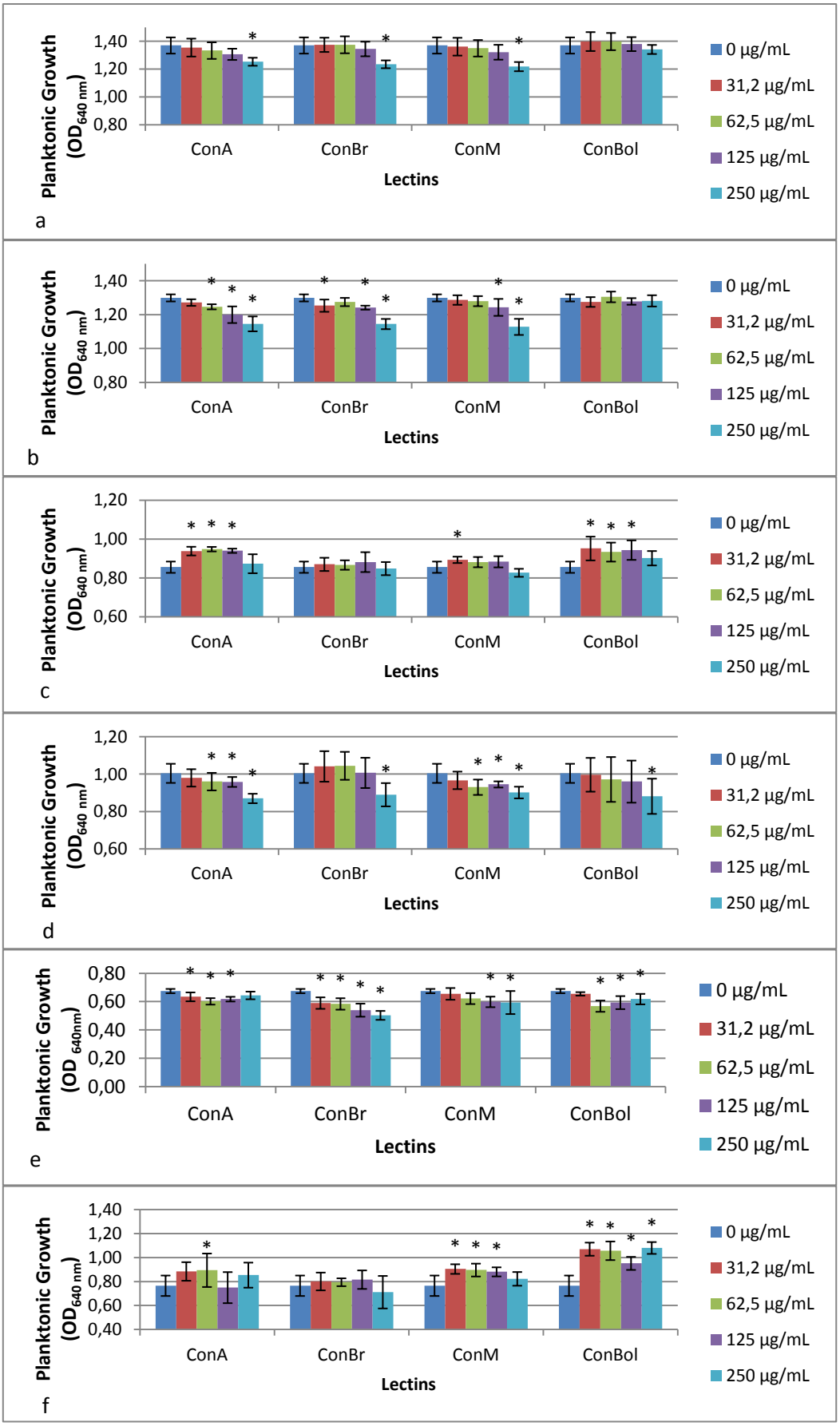

Figure 8. Effect of lectins on bacteria and yeast planktonic growth (a – *Pseudomonas aeruginosa*; b - *Klebsiella oxytoca*; c – *Staphylococcus aureus*; d - *Staphylococcus epidermidis*; e – *Candida albicans*; f – *Candida tropicalis*). *Statistically different from the control, 0 µg/mL ($p < 0.05$).

3.2. Bacteria and yeast early-stage adhesion

To verify the effect of lectins on bacteria and yeast early-stage adhesion (Figure 9), microbial cells were put in contact with the range of concentrations of all lectins, during 2 h. After that, the biomass was quantified by CV, being the quantitative analysis performed through the measurement of the optical density at 570 nm.

For *P. aeruginosa* (Figure 9a) when concentration of lectins was 250 μg/mL there was an increase of cell adhesion ($p<0.05$). However, lower concentrations of ConM and ConBol reduced it. In the case of *K. oxytoca* (Figure 9b) all lectins promoted an increase of cell adhesion ($p<0.05$).

Cell adhesion of Gram-positive bacteria (Figure 9c and 9d) decreased in the presence of lectins ($p<0.05$). Conversely, *C. albicans* and *C. tropicalis* (Figure 9e and 9f) adhesion was enhanced ($p<0.05$). There was an exception for ConM (125 and 250 μg/mL), when in contact with *C. tropicalis*, being observed a reduction ($p<0.05$) of cell adhesion.

3.3. Bacteria and yeast biofilm mass

The interference of the presence of the lectins in the establishment of biofilms (24 h) was also assessed. For that, bacteria and yeast biofilm mass was quantified by CV staining, being quantitative analysis performed through the measurement of optical density at 570 nm (Figure 10).

Biomass of *P. aeruginosa* (Figure 10a) decreased ($p<0.05$) in the presence of higher concentrations of ConBr and ConM, and intermediate concentration of ConBol. In contrast, for *K. oxytoca* (Figure 10b) all lectin enhanced ($p<0.05$) biofilm mass.

ConBol was the only lectin to promote a decrease ($p<0.05$) of *S. aureus* biofilm mass (Figure 10c). But, the other lectins had no significant effect ($p>0.05$), compared with the control. Interestingly, the same lectins (ConA, ConBr and ConM) promoted a decrease ($p<0.05$) of *S. epidermidis* biofilm mass (Figure 10d), in lower concentrations. But, on the other hand, for higher concentrations of ConBr, ConM and ConBol there was an increase ($p<0.05$) of biofilm mass.

In the case of *C. albicans* (Figure 10e), biofilm mass increased ($p<0.05$) for all concentrations and for all lectins, compared to the control.

For *C. tropicalis* (Figure 10f), ConA (31.2 and 62.5 μg/mL), ConBr (125 and 250 μg/mL) and ConBol (125 μg/mL) showed inhibitory ($p<0.05$) activity. In contrast, ConA (250 μg/mL), ConBr (31.2 μg/mL), ConM (62.5 and 250 μg/mL) and ConBol (31.2 μg/mL) showed a stimulatory ($p<0.05$) effect.

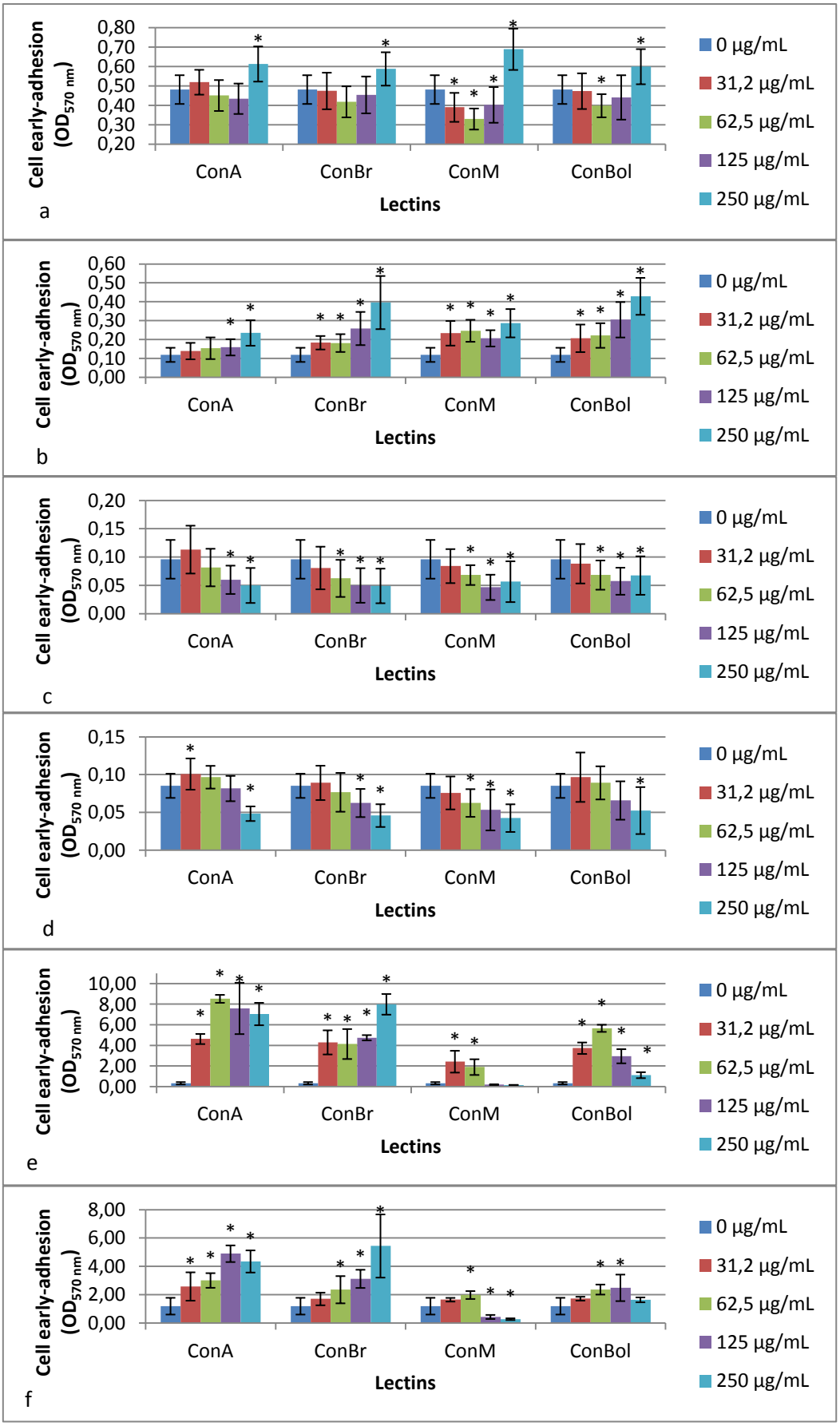

Figure 9. Effect of lectins on bacteria and yeast early-stage adhesion (a – *Pseudomonas aeruginosa*; b - *Klebsiella oxytoca*; c – *Staphylococcus aureus*; d - *Staphylococcus epidermidis*; e – *Candida albicans*; f – *Candida tropicalis*). *Statistically different from the control, 0 µg/mL ($p < 0.05$).

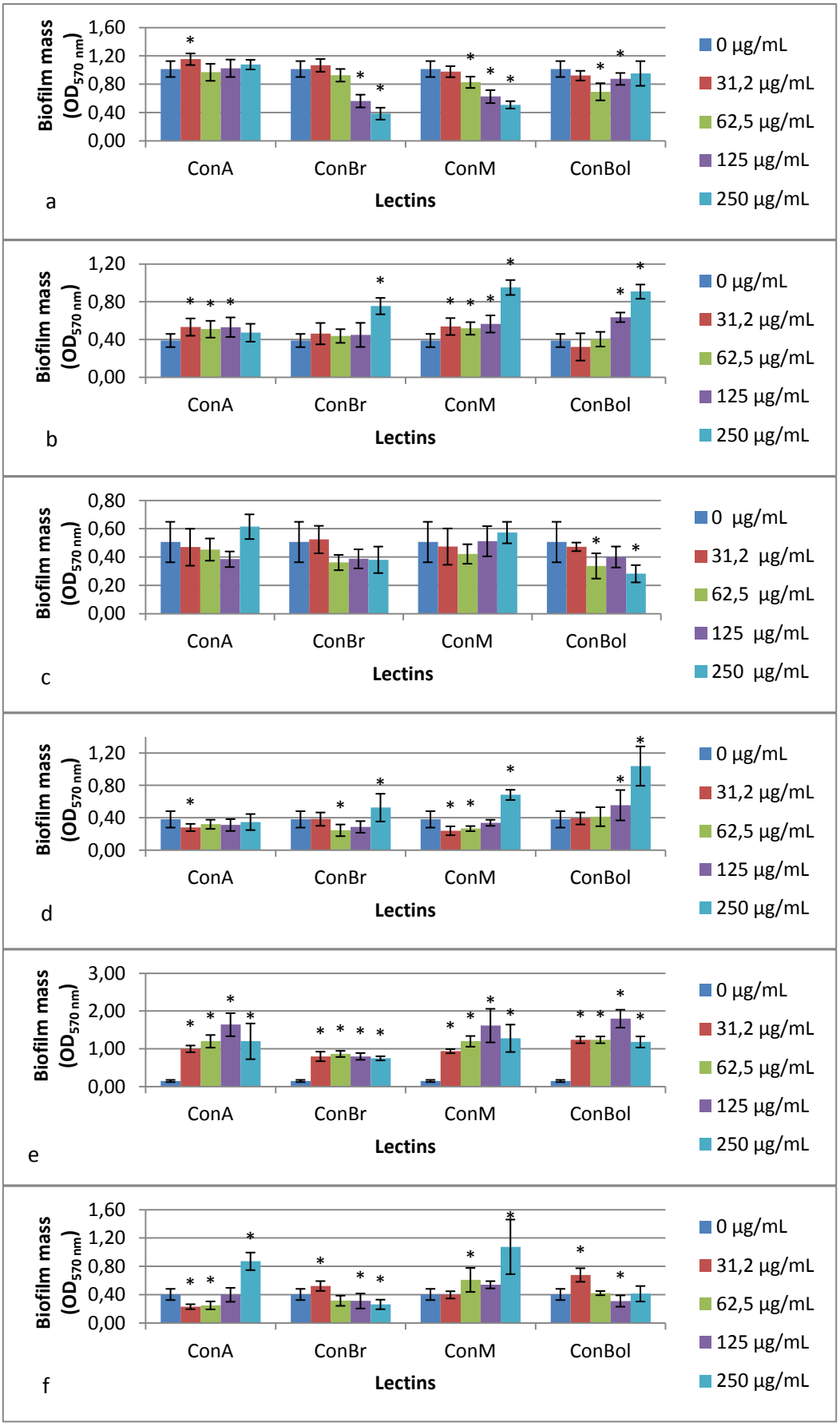

Figure 10. Bacteria and yeast biofilm formation (quantification of biofilm mass) in the presence of lectins (a – *Pseudomonas aeruginosa*; b - *Klebsiella oxytoca*; c – *Staphylococcus aureus*; d - *Staphylococcus epidermidis*; e – *Candida albicans*; f – *Candida tropicalis*). *Statistically different from the control, 0 µg/mL ($p < 0.05$).

3.4. Bacteria and yeast viable biofilm-entrapped cells

After 24 h of biofilm formation by bacteria and yeasts, the number of viable biofilm-entrapped cells was also determined (Figure 11).

An inhibitory effect ($p<0.05$) was observed for *P. aeruginosa* (Figure 11a), in the presence of 62.5 µg/mL of ConBol, whereas the other lectins had no effect ($p>0.05$). On the other hand, stimulatory effects ($p<0.05$) were found on *K. oxytoca*, in the presence of all studied lectins (Figure 11b).

Similarly, there was a stimulatory effect ($p<0.05$) in the case of *S. aureus* (Figure 11c), whereas on *S. epidermidis* (Figure 11d) only ConBol promoted this effect. The other lectins had no effect on *S. epidermidis* ($p>0.05$).

Regarding *C. albicans* (Figure 11e), ConA, ConBr and ConM induced a decrease ($p<0.05$) on the number of CFU, whereas ConBol had no significant effect ($p>0.05$). For *C. tropicalis* (Figure 11f), only ConM had no significant effect ($p>0.05$), compared to the control. The other lectins showed inhibitory activity ($p<0.05$).

3.5. Summary table of results

To better comprehend the results, it was created a table (Table 3) where they are represented in a condensed way. The effect of lectins on cells is represented by arrows. The direction of the arrows indicates the existence of an increase or inhibition of the condition which is being analyzed.

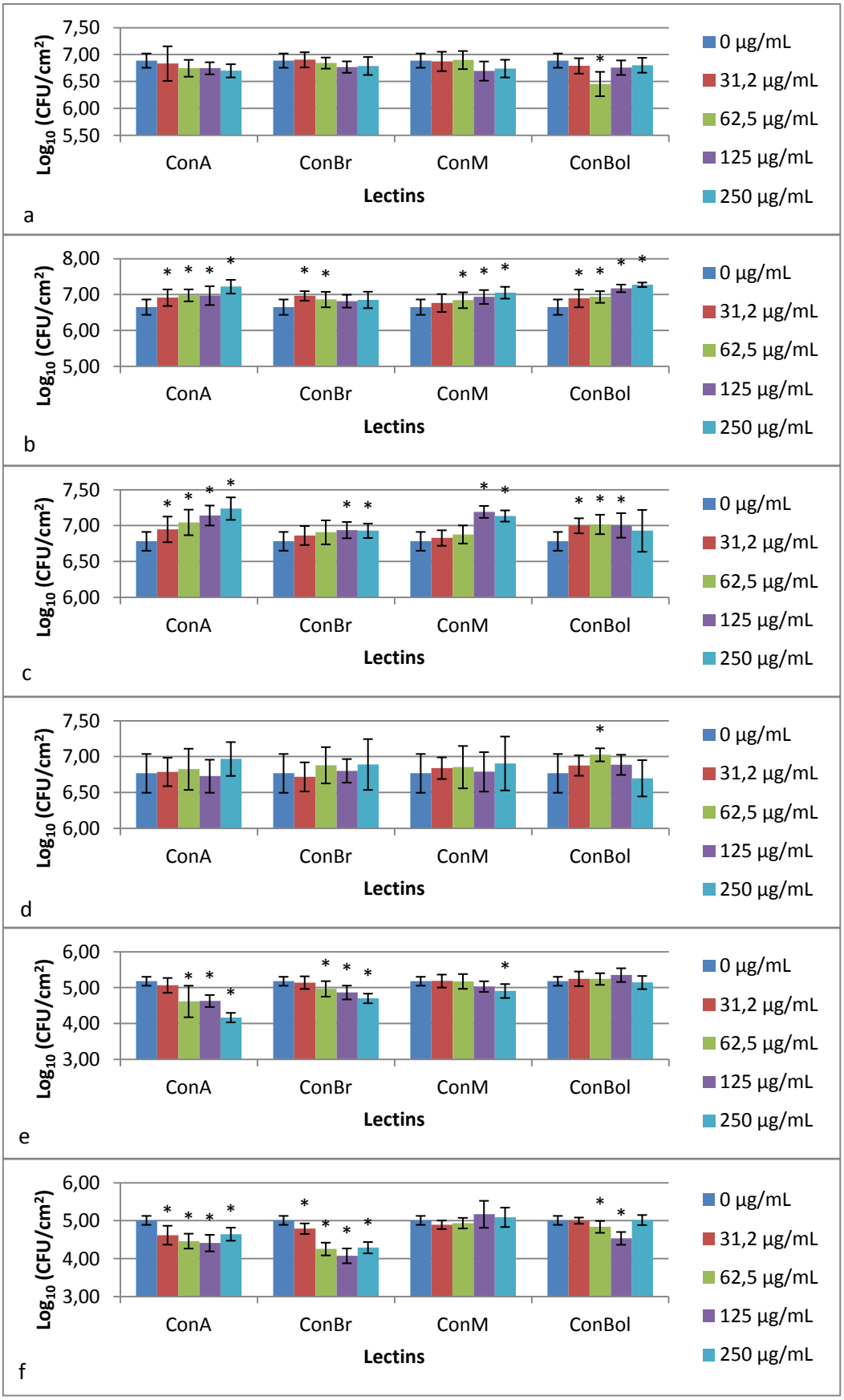

Figure 11. Bacteria and yeast biofilm formation (determination of the number of viable biofilm-entrapped cells) in the presence of lectins (a – *Pseudomonas aeruginosa*; b - *Klebsiella oxytoca*; c – *Staphylococcus aureus*; d - *Staphylococcus epidermidis*; e – *Candida albicans*; f – *Candida tropicalis*). *Statistically different from the control, 0 µg/mL ($p < 0.05$).

Table 1. Summary table of results

	Planktonic Growth				Early-stage adhesion				Biofilm mass				Viable biofilm-entrapped cells			
	ConA	**ConBr**	**ConM**	**ConBol**	**ConA**	**ConBr**	**ConM**	**ConBol**	**ConA**	**ConBr**	**ConM**	**ConBol**	**ConA**	**ConBr**	**ConM**	**ConBol**
P. aeruginosa	↓	↓	↓	–	↑	↑	↓	↑	↑	↓	↓	↓	–	–	–	↓
K. oxytoca	↓	↓	↓	–	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
S. aureus	↑	–	↑	↑	↓	↓	↓	↓	–	–	–	↓	↑	↑	↑	↑
S. epidermidis	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	–	–	–	↑
C. albicans	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	–
C. tropicalis	↑	–	↑	↑	↑	↑	↓	↑	↓	↓	↑	↑	↓	↓	–	↓

Legend:

↑ - represents an increase;

↓ - represents a decrease;

– - had no effect;

Chapter 4 | Discussion

Antibacterial activity of lectins was expected, because mostly were isolated from storage parts of plants, suggesting their possible contribution to plant defense against pathogenic microorganisms and because they were used in folk medicine, for the treatments of diseases related with microbial infections (56) (58). In fact, when lectins bind to bacteria, agglutination and/or precipitation of bacteria can occur. This can influence bacterial motility (blocking) and afterwards can have an effect on their multiplication, inhibiting bacterial growth (56) (60). In the present study, the lectins tested did not show a significant inhibitory effect against the four bacteria studied. In fact, ConBol (Figure 8), for instances, had no effect on both Gram-negative bacteria (*P.aeruginosa* and *K. oxytoca*) and even increased the growth of *S. aureus*. Though, there was a small inhibition of the growth of bacteria with the high concentrations of the other lectins (250 µg/mL), except for *S.aureus*.

Moreover, the high concentration tested (250 µg/mL) was a quite high value when compared with similar studies. In fact, Santi-Gadelha and colleagues (77) demonstrated that AaL lectin (150 µg/mL) had antibacterial activity against Gram-positive (*Clavibacter michiganensis*) and Gam-negative (*Xanthomonas axonopodis*) bacteria. Furthermore, Oliveira and colleagues (58) verified that EuniSL lectin inhibited the growth of *S. aureus*, *P. aeruginosa* and *Klebsiela sp*. with a minimum inhibitory concentration (MIC) of 1.5 µg/mL and inhibited the growth of *B. subtilis*, *Streptococcus sp*. and *E. coli* with a MIC of 16.5 µg/mL. However, Cavalcante and colleagues (80), when tested antimicrobial action of lectins from Diocleinae subtribe, have verified the need of a concentration of 500 µg/mL to inhibit *S. mutans* growth.

Mostly microorganisms express surface-exposed carbohydrates and each one is a potential lectin-reactive site. The carbohydrates can be covalently bound, as in glycosylated teichoic acids to peptidoglycan, or non-covalently bound, as in capsular polysaccharides (77) (80). So, the differences that exist on surface-exposed carbohydrates of bacteria are responsible for the recognition of bacteria by lectins (80) (81).

From the results obtained, it is possible to see that *K. oxytoca* is more sensitive than *P. aeruginosa*, because its growth was affected by lower concentrations of lectins. Moreover, results indicate an inhibition on *S. epidermidis* planktonic growth but not on the *S. aureu*s (both Gram-positive bacteria). These differences between species, more evident than between Gram type, could be attributed to the different surface carbohydrates present on the different bacteria cell wall.

All lectins used in this study are members of Diocleinae (Leguminosae) subfamily, being highly homologous (9) (67).

Through analysis of Figure 8, it can be observed that although ConA and ConBr presented a similar effect on *P. aeruginosa*, *K. oxytoca*, *S. epidermidis* and C*. albicans*. For *S.*

aureus and *C. tropicalis*, ConA lectin stimulated the growth, whereas ConBr had no significant effect. These lectins are highly homologous, but they have distinct biological activities. ConBr had differences in quaternary structure in relation to ConA (quaternary structure is more open for ConBr than for ConA) that may constitute the structural basis for explaining their different biological activities (66) (67) (89). The quaternary structure of Diocleinae lectins has relevant aspects as the subunit multivalency and the relative orientation of the carbohydrate-binding sites, that may explain their distinct biological activities (66). Besides variations on structure, the lectin ConBr may have not significant activity due the absence of proper ConBr glycoconjugates targets in the bacteria/fungal cell wall or cellular membrane, or inaccessibility of the lectin to the target (90).

Lectins promoted an inhibition of the *C. albicans* growth, as observed on Figure 8. Plant lectins, such as lectins from *Pisum sativum* and from *Capsicum annuum*, revealed to be able to inhibit yeasts growth. Lectin from *Pisum sativum* was able to inhibit fungi like *Aspergillus flavus* and *Trichoderma viride* and lectin from *Capsicum annuum* was able to inhibit *Aspergillus niger* and *Fuscarium solani*, among others (81) (83). In contrast, lectins promoted a stimulation of *C. tropicalis* (Figure 8). So, subtle structural differences in the surface glycoconjugates of these fungi species can affect the binding specificity of lectins (90). Antifungal activity was related to the lectin surface cell wall carbohydrate binding property, affecting activity and viability of fungal cells (81). Additionally, antifungal activity of some proteins or peptides was associated with chitin binding property (84). This binding property can simulate the carbohydrate binding property since chitin is composed of modified glucose subunits (N-acetyl glucose amine). Almost all lectins that bind glucose, equally recognize and interact with N-acetylglucosamine and even the acetamide group can increase the binding activity. Fungal cell wall can disrupt, increasing toxicity, when chitin binding occur, since chitin (major component of fungal wall) is a polymer of N-acetylglucosamine (81). Antifungal activity is important since, to date, only a small number of plant lectins have been described to really have this activity (57).

Regarding adhesion (Figure 9), lectins had inhibitory effects on cell adherence for Gram-positive bacteria. Teixeira and colleagues (9) verified that the adhesion of some *Streptococcus* species (Gram-positive bacteria), such as *Streptococcus mutans*, *Streptococcus oralis* and *Streptococcus mitis* was inhibited by lectins like ConA, ConBr, lectins from *Dioclea violacea* (DVL) and from *Dioclea grandiflora* (DGL). Some concentrations of ConM and ConBol had the same inhibitory effect for *P. aeruginosa* (Figure 9a) and *C. tropicalis* (Figure 9f). The specific lectin-carbohydrate interaction between bacteria or fungal is one of the many molecular mechanisms involved in adherence of microorganisms, and consequent

development of biofilms. Specific ligands on the microorganisms' surfaces appear to interact through diverse mechanisms, including cell-cell and substrate-cell interactions (61). Lectin binding on the microbial wall may block the available sites for exopolysaccharides, as well cell-cell binding, and thus reduce attachment to the surface. Lectins can be used, being present in the milieu, to prevent the long-term attachment of bacteria on surfaces, and the consequent biofilm formation, because lectins are able to agglutinate bacteria (60).

Biofilms are a structured community of surface-associated microbial cells enclosed in a self-produced polymeric matrix, forming a sessile population (1) (37) (54) (91).

Inside biofilms, cells undergo physiological changes in relation to individual planktonic cells. The extracellular matrix is composed, essentially, by exopolysaccharides and it is believed that it plays a key role in biofilm resistance (92) (93). EPS (mostly exopolysaccharides) are responsible for cell-cell and cell-surface interactions (94).

Through analysis of Figure 10, it can be observed that the tested lectins promoted a decrease on biofilm mass of *P. aeruginosa*. Lectins from *Marasmium oreades* (MOA) and from *Hippeastrum hybrid* (HHA) have the capacity to bind, respectively, to galactose and mannose of the PsI (Polysaccharide synthesis locus) exopolysaccharide, which contributes to biofilm formation by *P. aeruginosa*. PsI is essential for bacterial adherence to a substratum (promote cell-cell and cell-surface interactions) and maintenance of biofim structure (95) (96).

Although ConBr, ConM and ConBol promoted a decrease of biofilm mass for *P. aeruginosa*, as well as ConBol for *S. aureus*, there was no reduction of the viability of these bacteria (Figure 11 a and c). Islam and colleagues (60) verified an arrangement modification and absence of exopolysaccharide matrix when biofilms of *S. mutans* were treated with a lectin from *Trigonella foenumgraecum* (TFA). Their work revealed that cell-surface molecules, where lectins bind, can be involved in the maintenance of the arrangement and structural integrity of biofilms. Inhibition of adhesion and of consequent biofilm formation without affecting bacteria cells viability may prevent selective pressure and the development of microbial resistance (61).

When lectins induce an enhancement of cell adhesion and biofilm biomass, but a decrease in the number of viable cells (Figures 9, 10, 11 e and f), it seems that lectins act by inducing a physiological change on cells rather than by a direct action on cells binding (92). Peumans and Van Damme (62) reported that cells of the intestine are capable of endocytosed PHA lectin. This lectin induces, when it enters the cells, an enhancement of the metabolic activity of the cells. Further studies should be performed to verify whether lectins are capable of being endocytosed by microbial cells and modify their metabolic activity. If this happens,

some phenomena, as enhancement of cell growth and biofilm development in the presence of lectins, will be probably better understood.

Chapter 5 | Conclusion and suggestions for future research

This study was conducted to verify whether dioclinae lectins had some effect on some bacteria and yeasts which are important nosocomial agents.

The effect of lectins on microorganisms planktonic growth, early-stage adhesion and biofilm formation, inspected by, respectively, turbidimetry, quantification of adhered cells, biofilm biomass and the cultivate biofilm-growing cells, was evaluated.

Through careful analysis of the results, it can be concluded that the tested lectins did not show a robust inhibitory effect against the microorganisms studied, being their activities different between species. The less significant antimicrobial activity occurred on *K. oxytoca*, because only its planktonic growth was inhibited by lectins.

For Gram-positive bacteria, early-stage cell adhesion decreased in the presence of lectins, however enhanced for *Candida* species.

The viability of the cultivated bacteria cells of the biofilms were not affected by lectins, whereas yeasts biofilm cells demonstrated to lose viability after exposure to lectins.

Despite lectins demonstrate antimicrobial activity, it was also observed that some of them, for some microorganisms, promoted a stimulatory effect of growth, adhesion and biofilm formation.

Being lectins natural compounds with antimicrobial properties and high specificity, a continuous research to understand all mechanisms of their action is really important.

So, research should be performed in order to verify whether lectins are capable of being endocytosed by microbial cells and modify their metabolic activity.

To explore more exhaustively the potential action of lectins, an interesting approach using several lectins at the same time or lectins combined with conventional antibacterial and antifungal agents could be tested to verify whether there are some synergic effects. The use of several lectins at the same time will help to understand the interaction between lectins, when they act together.

Additionally, microbial cells wall may be coated with mannose/glucose (specific monosaccharide for lectins used) to verify if there are an enhancement of lectin activity.

The development of resistance to lectins or cross-resistance to other antimicrobial agents should be also studied.

Further investigations must address the toxicity of these lectins regarding humans, for further applications on heath sector.

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